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(54) Title: METHOD FOR PRODUCTION OF PREDETERMINED POLYRIBONUCLEOTIDES (57) Abstract Methods of production of predetermined polyribonucleotide. These methods may then be used to synthesize RNA coding for a desired protein. The methods described include: (a) adding a single nucleotide on to the 3' end of a recipient ribonucleotide of predetermined composition by reacting the compound of the form AppNp with the recipient ribonucleotide in the presence of T4 RNA ligase and isolating the extended ribonucleotide where N is a ribonucleotide selected from the group adenine, guanine, cytosine, uracil and inosine. (b) adding a ribonucleotide of at least two bases by phosphorylating a donor ribonucleotide of at least two bases at the terminal 5' and 3' ends, then mixing the phosphorylated donor ribonucleotide with a recipient ribonucleotide in the presence of T4 RNA ligase to add the donor onto the 3' end of the recipient ribonucleotide where the recipient nucleotide is selected from the group consisting of a dinucleotide phosphorylated in the free 5' position and a nucleotide longer than 2 base pairs with either a hydroxyl or a phosphate group on the 5' terminus and a hydroxylated 3' end. (c) synthesizing ribonucleic acid of a predetermined composition by sequentially building polynucleotides using the above methods.		

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1
METHOD FOR PRODUCTION OF PREDETERMINED POLYRIBONUCLEOTIDES
BACKGROUND AND PRIOR ART

There are three general steps involved in the production of a protein utilizing genetic engineering techniques. The first step includes laboratory production of or isolation and purification of the desired gene which codes for a particular protein. The second step is the recombination of the gene with a proper transfer vector such as a plasmid. The third step includes transferring the recombined vector into a particular microorganism and inducing the microorganism to produce the particular gene product.

The present invention is directed towards a method of accomplishing the first step. Current methodology for in vitro production of genes by sequential addition of nucleotides consists of using chemical techniques (Itakura, K. and Riggs, A., Science 109:1401 (1980), (Khorana, H.G., Science 203:614 (1979), enzymatic procedures (S. Gillam, P. Jahnke, C. Astell, S. Phillips, C.A. Hutchinson, M. Smith, Nucleic Acids Res. 6,2973 (1979) and solid phase techniques to produce the desired DNA or RNA molecules. The chemical procedures of DNA synthesis are tedious because the reactions involved are non-specific such that extensive purification of the desired product is necessary after each operative step. Thus, synthesis of RNA and DNA by chemical techniques is significantly less desirable than enzymatic systems because greater amounts of starting material are required to produce comparable yields thereby increasing costs, the purification techniques are both time consuming and wasteful where some product is lost at each step, and the large number of steps involved in blocking and unblocking reactive sites provide many opportunities for error and risk of contamination by degradative enzymes which destroy the synthesized product.

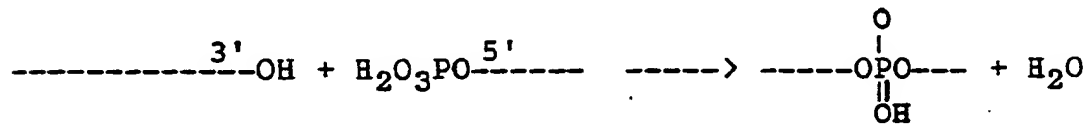
Enzymatic synthesis of RNA using polynucleotide phosphorylase and T4 RNA ligase have been reported. The polynucleotide phosphorylase method is limited to production of oligodeoxynucleotides. Under controlled conditions, poly-

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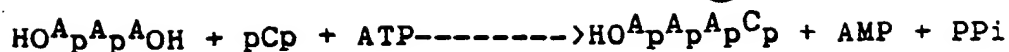
nucleotide phosphorylase adds predominantly a single nucleotide to a short oligodeoxynucleotide which is then isolated by chromatographic techniques. Solid phase techniques are performed by binding the nucleotide chain to a solid support material and using the above chemical methodology to add nucleotides stepwise.

The present invention teaches the composition and method of production of a compound useful in the process of synthesizing a predetermined sequence of RNA using the enzyme T4 RNA ligase. Processes for transcribing the synthesized RNA into DNA so that the synthesized gene may be incorporated into a plasmid, inserted into a microorganism and expressed, are known in the art.

The existence, purification and mechanism of the enzyme T4 RNA ligase which is isolated from *Escherichia coli* infected with bacteriophage T4 have been described (Silber, R., Malathi, V.G. & Hurwitz, J., Proc. Natl. Acad. Sci. (1972) 69:3009). This enzyme catalyzes the formation of a phosphodiester bond between the phosphate group on the 5' end of a donor nucleotide and the hydroxyl group on the 3' end of the recipient oligonucleotide as shown below.



Japanese Patent Application 1980-19003 (published February 9, 1980 teaches the utilization of T4 RNA ligase to extend a polynucleotide by adding a single mononucleoside diphosphates (pNp) onto the 3' end of a nucleotide sequence with no terminal phosphate groups. This methodology requires as the starting substrate a trinucleotide, which must either be obtained commercially or synthesized by chemical means, and requires adenosine triphosphate (ATP) as an energy source for the reaction. Additionally, the invention teaches only the addition of a single base and is not capable of producing oligo or polynucleotides for which sequential addition is required.



Abbreviations to be used in this application for convenience are provided in Table 1.

TABLE 1

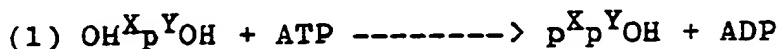
<u>Abbreviation</u>	<u>Definition</u>
A	Adenosine
C	Cytosine
G	Guanosine
U	Uridine
N,X,Y,Z	any ribonucleotide
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
BAP	Bacterial alkaline phosphatase
VPD	Venom phosphodiesterase
AppNp	A ^{5'} p ^{5'} pN ^{3'} p (see text for further explanation)
>p	2' - 2' cyclized phosphodiester bond
ul	Microliter
nm	Nanometer
TEAB	Triethylammonium Bicarbonate Buffer
DTT	DL-Dithiothreitol (Cleland's reagent)
PNK	Polynucleotide kinase
nbzl	o-nitrobenzyl group
pnbzl	o-nitrobenzyl phosphate group
UV	Ultraviolet light
MOPS	3-[N-morpholino] propanesulfonic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

This invention teaches the synthesis of biologically active RNA and DNA molecules. All nucleotide sequences are indicated by their abbreviations according to Table 1. All phosphodiester linkages between nucleotides are 3'-5' unless otherwise indicated.

SUMMARY OF THE INVENTION

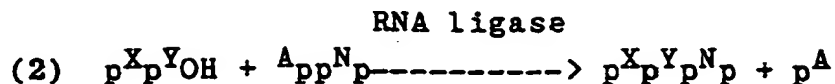
The invention disclosed herein teaches methods of synthesizing single stranded RNA molecules of pre determined base sequence using the enzyme T4 RNA ligase to specifically add a mono-, di-, oligo- or polynucleotide (the donor) onto the 3' end of a ribonucleotide sequence two bases or longer (the recipient) in a 5'--3' linkage. The shortest recipient which the T4 RNA ligase will act upon in the catalysis is a dinucleotide phosphorylated in the 5' position. If the recipient is a trinucleotide or longer, the 5' terminal end may be either phosphorylated or hydroxylated to allow specific addition of the donor to the 3' end of the recipient. Referring to the method for adding a single base as a donor onto the end of a recipient, if the starting material for the recipient is a dinucleotide either obtained commercially or synthesized by chemical techniques known in the art, the first step is to phosphorylate the free 5' end. This step is performed by mixing the dinucleotide with ATP in the presence of the enzyme polynucleotide kinase (PNK) (Richardson, C.C., Proc. Natl. Acad. Sci. U.S.A. 54, 158 (1965) which specifically phosphorylates the 5' terminus of a nucleotide as depicted in equation 1. If the recipient is a trinucleotide or longer, this phosphorylation step is not necessary.

PNK

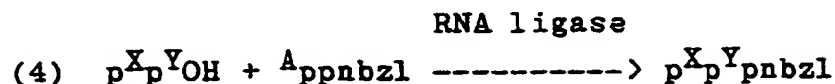
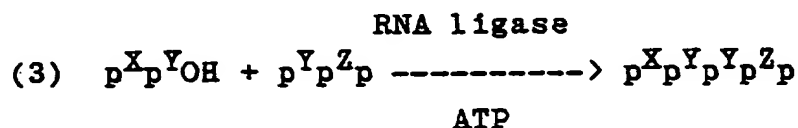


The second step is to mix the recipient dinucleotide with the chemical compound $\text{p}^1\text{-Adenosine}$, $\text{p}^2\text{-(3' nucleotide monophosphate)-5' pyrophosphate}$ of the form (AppNp), where N is a ribonucleotide selected from the group adenine, cytosine, guanine, uracil, and inosine. The compound AppNp and its synthesis are disclosed in the copending application (____). The reaction of the known recipient ribonucleotide with the AppNp in the presence of RNA ligase

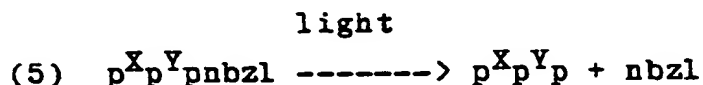
results in the production of a nucleotide as depicted in equation 2. This reaction does not require ATP as an energy source.



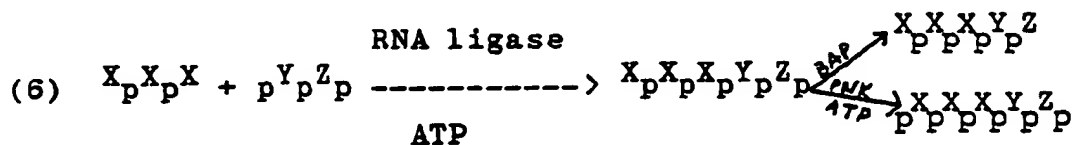
This invention also discloses a method of adding a donor RNA molecule of 2 bases or longer to a recipient ribonucleotide such that 5' phosphate on the donor binds specifically with the 3' hydroxyl group of the recipient in the presence of ATP and RNA ligase to form an extended ribonucleotide (equation 3). The phosphate group on the 3' end of the donor molecule makes that end unreactive to RNA ligase so as to prevent further addition of the donor molecules. Again for this method, if the recipient is a dinucleotide it must be phosphorylated on the 5' end whereas if it is a trinucleotide or longer, phosphorylation is unnecessary (equation 4).



To phosphorylate the 3' end of the donor molecule, the 5' phosphorylated dinucleotide is reacted with the compound Appnbzl in the presence of RNA ligase (equation 4). The nbzl moiety may be removed from the resulting nucleotide by exposure to strong light (equation 5).



The resulting extended ribonucleotide can then be treated with bacterial alkaline phosphatase (BAP) which cleaves terminal phosphate groups preparing it to act as a recipient or it can be treated with polynucleotide kinase (PNK) phosphorylating the 5' end thereby converting it to a donor (equation 6).

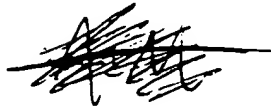


The reactions described hereinabove are examples of a number of methods for production of nucleotides. These methods may be used effectively on longer oligonucleotides as well as the dinucleotides described in this disclosure. In this way, entire RNA molecules coding for particular proteins may be synthesized.

For each of the reactions described below, it is necessary to determine the identity of the reaction products. Identification of reaction products throughout these examples is made by taking ratios of absorption at 250, 260 and 280 nm and comparing them to known standards. Identification and purity are also assayed by paper electrophoresis at pH 5.0 in sodium citrate buffer and at pH 2.8 in ammonium formate buffer at 800 volts for 1 hour.

Description of the Specific Embodiments

The present invention teaches ligase in the preparation of nucleic acid base sequence. As hereinbefore and hereinafter methods are disclosed. One method involves the addition of a donor mononucleotide to a recipient nucleotide having at least two bases. The other method involves the addition of donor nucleotides having two bases. As to which of these methods show the composition of the nucleotide and the availability of commercially used as starting material.



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Monoribonucleotide Additions

In the synthesis of a trinucleotide of predetermined base sequence from a known dinucleotide and an AppNp compound, it is first necessary to phosphorylate the free 5' end of the dinucleotide. This is accomplished using the enzyme polynucleotide kinase under conditions known in the art.

Following the phosphorylation step, it is necessary to isolate the phosphorylated dinucleotide from the other components of the reaction mixture. This is achieved by fractionating the reaction mixture and treating the pooled fractions containing the phosphorylated dinucleotide with ATPase which cleaves a pyrophosphate from ATP contaminating the phosphorylated dinucleotide pool. Isolation of the phosphorylated dinucleotide by fractionation is then possible. The ATPase used in Example 1 below was obtained and purified as a side product of the T4 RNA ligase purification however, any ATPase should be effective.

The phosphorylated dinucleotide or other form of recipient nucleotide is then incubated with an AppNp compound where the specific compound to be used is one for which N is the nucleotide to be added to the 3' end of the

dinucleotide. The preferred reaction conditions are provided in the examples below.

Finally, the resultant nucleotide may be treated with a phosphatase which removes terminal phosphates leaving hydroxyl groups on the 5' and 3' termini. The conditions for the phosphatase reactions, dependent on which phosphatase is used, are well-known in the art. The phosphatase treated nucleotide is then prepared for subsequent additions as described below.

Additions of More Than One Nucleotide

Addition of donor ribonucleotides of two bases or more can be made to the 3' end of a recipient ribonucleotide by phosphorylating the 5' and 3' ends of said donor and incubating said phosphorylated donor with said recipient in the presence of RNA ligase and ATP. The recipient ribonucleotide for this reaction must be a 5' phosphorylated dinucleotide or an oligonucleotide with a phosphorylated or free 5' terminus.

To phosphorylate the 3' end of the donor ribonucleotide, the ribonucleotide is incubated with Appnbzl in the presence of RNA ligase, thereby bonding the pnbzl moiety to the 3' end. The reaction mixture is boiled to inactivate the RNA ligase and then mixed with ATP in the presence of polynucleotide kinase to phosphorylate the 5' end of said donor molecule.

The donor is isolated and may then be exposed to strong light such as 300 watt mercury vapor lamp to remove the nbzl moiety leaving the donor phosphorylate at the 5' and 3' ends. The donor may be added to the recipient with or without removing the nbzl moiety.

Following the addition of the donor ribonucleotide to the recipient, the resultant extended nucleotide may be treated with a phosphatase to remove all terminal phosphate groups thereby converting it to a form wherein it may act as a recipient. Alternatively, the extended nucleotide may be

treated with PNK to phosphorylate the 5' end and allow it to be used as a donor.

It is anticipated that all purification steps wherein the preferred method described is fractionation using a DEAE sephadex column may also be achieved by high pressure liquid chromatography, thin layer chromatography, gel and paper electrophoresis, and other known separation techniques without departing from the scope of this invention.

EXAMPLE I

Synthesis of ApGpA

Adenylyl (3' - 5') guanosine (ApG) is first mixed with ATP in the presence of polynucleotide kinase to form pApG. The reaction mixture containing 70 ul of 15 mM AG (SIGMA), 60 ul of 1M Tris-HCl (pH 8.1), 10 ul 2-mercaptoethanol, 100 ul of 100 mM $MgCl_2$, 150 ul of 20 mM ATP, 50 ul H_2O , and 100 ul PNK (1500 units/ml) was incubated at 37° for 1 hour then fractionated by a DEAE Sephadex A-25 column on a linear gradient of 0.2 to 0.8 M TEAB (pH 7.6). The fourth peak containing ATP and pApG as identified by UV absorption is pooled, brought to dryness with methanol under a vacuum, and then lyophilized.

To separate the ATP from the pApG the pooled fraction is treated with ATPase and fractionated on a column. 200 ul of the pApG/ATP mixture ($A_{260}=49.2$), 100 ul of 100mM DTT, 100 ul of 1M MOPS pH 7.9, 150 ul of 100 mM $MgCl_2$, 800 ul of H_2O and 250 ul of T4 ATPase (5000 units/ml) are mixed and incubated for 1 hour at 37°C. The resulting product was fractionated on a DEAE Sephadex A-25 column on a 0.2 - 0.8 M TEAB (pH 7.6) linear gradient. The second major peak at 260 nm, the pApG fraction, was pooled.

The pApG from the above reaction was then used in the synthesis of pApGpAp. T4 RNA ligase was obtained using the procedure of Silber et. al. 112 ul pApG (.87 mM), 58 ul AppAp (3.4 mM), 15 ul 1M HEPES pH 7.5 and 50 ul RNA ligase (1500 units/ml) were mixed and incubated for 1 hour at room

temperature. The resultant mixture was fractionated on DEAE Sephadex A-25, and the pApGpAp peak was identified by paper electrophoresis at pH 5.0 in 50 mM sodium citrate buffer. The pooled pApGpAp fraction was brought to dryness in the presence of methanol under vacuum and lyophilized.

The sample was then treated with bacterial alkaline phosphatase (BAP). 40 μ l pApGpAp, (approximately 1.0 unit at 260 nm.), 5 μ l 1M Tris-HCl pH 8.1, 5 μ l BAP C (200 units/ml, Worthington Biochemical), and 50 μ l H₂O was incubated for 30 min at 65°C and fractionated on a DEAE Sephadex A-25 column with a 0.1 to 1.0 M TEAB pH 7.6 linear gradient. The ApGpA containing fractions were pooled.

EXAMPLE 2

Synthesis of pGUp from GAU

The trinucleotide GAU (Collaborative Research) was assayed for purity by electrophoresis. A reaction mixture containing 225 μ l 26 mM Appnbzl, 300 μ l of 3.3 mM GpApU, 75 μ l 1 M MOPS pH 7.9, 300 μ l 0.1M MgCl₂, 150 μ l 100 mM DTT, 120 μ l H₂O, 180 μ l RNA ligase and 150 μ l 100% dimethyl sulfoxide DMSO were incubated at room temperature for 20 hours. The reaction mixture was then boiled for two minutes and 1400 μ l of the boiled mixture was then mixed with 300 μ l of 20 mM ATP, 200 μ l of 100 mM DTT, and 100 μ l PNK (1500 units/ml), and incubated for 90 minutes at 37°C.

The reaction mixture was then fractionated on a DEAE Sephadex A-25 column with linear gradient of 0.1 M to 1.0 M TEAB pH 7.6. The fourth peak containing the pGpApUpnbzl was pooled and then exposed to a 300 watt mercury vapor lamp at a distance of 5 cm for 15 minutes in a water jacketed cooling chamber.

EXAMPLE

The reaction mixture consisting of 34 ul of 3.3 mM AAA (Boehringer) 70 ul of 1.0 mM pGpApnbzl (synthesized by the method of EXAMPLE 2), 40 ul of 1.0 M HEPES pH 7.83, 5 ul of 1.0 M MgCl_2 , 25 ul of 20 mM ATP (SIGMA), 2 ul of bovine serum albumin (BSA) (1 mg/ml), 40 ul of RNA ligase (1500 units/ml), and 20 ul of 100% dimethyl sulfoxide was incubated overnight at room temperature. The reaction mixture was fractionated on a DEAE Sephadex A-25 column. The sixth major peak with absorption ratios of $250/260 = 1.17$ and $280/260 = .667$ was identified as the product AAAGApnbzl. The oligonucleotide was then exposed to the mercury vapor lamp as described in EXAMPLE 2 to remove the nbzl group.

EXAMPLE 4

The reaction mixture containing 75 ul of (1.2 mM) ACCU (which may be synthesized using EXAMPLE 1), 90 ul of 1.0 mM pCCGAp (EXAMPLE 1), 30 ul of 1.0 M MOPS (pH 7.9), 120 ul of 100 mM MgCl_2 , 70 ul of 100 mM DTT, 60 ul of DMSO (100%), 60 ul of 20 mM ATP, 15 ul of BSA (10 mg/ml), 60 ul of RNA ligase (1500 units/ml) and 100 ul of H_2O was incubated at room temperature (approximately 24°) for 16 hours. The mixture was fractionated on a DEAE Sephadex A-24 column with a 0.1 to 2.0 M TEAB pH 7.5 linear gradient and the sixth peak identified by UV absorption as being the polynucleotide ApCpCpUpCpCpGpAp.

CLAIMS

1. The method of synthesizing RNA of predetermined base sequence using the enzyme T4 RNA ligase which comprises adding a donor specifically to the hydroxylated 3' end of a recipient of at least two bases whereby if said donor consists of two or more nucleotide bases, adenosine triphosphate is provided as an energy source for the addition.

2. The method of adding a predetermined single ribonucleotide to a recipient ribonucleotide of at least two bases which comprises:

mixing said recipient ribonucleotide with a compound of the form AppNp in the presence of T4 RNA ligase under conditions which favor transfer of the pNp moiety from the AppNp to said phosphorylated ribonucleotide at the 3' end;

wherein the N of the AppNp is a ribonucleotide selected from the group consisting of adenine, guanine, cytosine, uracil and inosine.

3. The method of adding a predetermined ribonucleotide of at least 2 bases to a recipient ribonucleotide of at least two bases comprising the steps of:

phosphorylating specifically the 3' end of the donor ribonucleotide;

phosphorylating the 5' end of the donor ribonucleotide;

incubating the recipient ribonucleotide with the phosphorylated donor ribonucleotide in the presence of T4 RNA ligase under conditions which permit covalent bonding between the recipient and donor ribonucleotide.

4. The method of Claim 2 wherein said recipient ribonucleotide is selected from the group consisting of a 5' phosphorylated 3' hydroxylated diribonucleotide, an oligonucleotide phosphorylated at the 5' end and hydroxylated at the 3' end, and an oligonucleotide hydroxylated at the 5' and 3' ends.

5. The method of Claim 3 wherein said recipient ribonucleotide is selected from the group consisting of a 5' phosphorylated 3' hydroxylated diribonucleotide, an oligonucleotide phosphorylated at the 5' end and hydroxylated at the 3' end, and an oligonucleotide hydroxylated at the 5' and 3' ends.

6. The method according to Claim 4 further comprising the step of synthesizing said 5' phosphorylated, 3' hydroxylated ribonucleotide by reacting a ribonucleotide hydroxylated at the free 3' and 5' termini with adenosine triphosphate (ATP) in the presence of polynucleotide kinase.

7. The method according to Claim 6 further comprising the steps of:

reacting a ribonucleotide hydroxylated at the free 3' and 5' termini with ATP in the presence of polynucleotide kinase to form a phosphorylated ribonucleotide;

fractionating the reaction mixture by an exchange column chromatography;

treating the portion of the fractionated reaction mixture containing the phosphorylated ribonucleotide with an ATPase;

fractionating the ATPase treated reaction mixture to isolate the phosphorylated ribonucleotide.

8. The method according to Claim 5 further comprising the step of phosphorylating specifically the 5' end of a recipient ribonucleotide by reacting said ribonucleotide with ATP in the presence of polynucleotide kinase.

9. The method according to Claim 3 wherein the step of phosphorylating the 5' end of the donor ribonucleotide comprises the step of reacting said ribonucleotide with ATP in the presence of polynucleotide kinase.

10. The method according to Claim 3 wherein the step of phosphorylating the 3' end of the donor ribonucleotide comprises the step of

reacting a ribonucleotide of at least two bases with the compound Appnbzl in the presence of T4 RNA Ligase under conditions which favor bonding of the pnbzl group to the 3' terminus of said ribonucleotide.

11. The method of synthesizing RNA of predetermined base sequence comprising the steps of:

a) phosphorylating a ribonucleotide of known base sequence of at least 2 bases at the 5' terminus;

b) reacting said RNA with AppNp in the presence of T4 RNA ligase to bond the pNp moiety of AppNp to the 3' terminus of said ribonucleotide;

c) treat the resulting extended RNA with an enzyme which cleaves terminal phosphate groups;

d) repeat steps b and c until the ribonucleic acid of predetermined sequence is produced;

wherein N is a ribonucleotide selected from the group adenine, guanine, cytosine, uracil and inosine.

12. The method of Claim 2 wherein said conditions under which the transfer of said pNp moiety is favored consists of incubation of said recipient ribonucleotide with said AppNp compound in the presence of RNA ligase in a small volume of HEPES Buffer for 1 hour at 37°C.

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ¹ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ³ C12P 19/34		
II. FIELDS SEARCHED Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
IPC:	C12P 19/34, C12N 9/00, C12N 9/12, C12 N1/00, C 07H 21/02	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
BIOSIS PREVIEWS 1969-83 BOYER, <u>THE ENZYMES</u> VOL. XV PT. B, 1982, ACADEMIC PRESS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	N, ENGLAND ET AL, BIOCHEMISTRY, VOLUME 17, NUMBER 11, 1978, PAGES 2069-2076.	1
X	N, KIRUCHI ET AL, PROC. NATL. ACAD. SCI. USA, VOLUME 75, NUMBER 3, 1978, PAGES 1270-1273.	2,4,11
X	N, OHTSUKA ET AL, CHEMICAL ABSTRACTS, VOLUME 90, 1979, PAGE 295, ABSTRACT NUMBER 182705X, NUCLEIC ACIDS RES., VOLUME 6, NUMBER 2, 1979, PAGES 443-454.	3,5,8,10
Y	N, ENGLAND ET AL, BIOCHEMISTRY, VOLUME 17, NUMBER 11, 1978, PAGES 2069-2076.	3,5-9,12
Y	N, KIRUCHI ET AL, PROC. NATL. ACAD. SCI. USA, VOLUME 75, NUMBER 3, 1978, PAGES 1270-1273.	6,7,12
Y	N, OHTSUKA ET AL, CHEMICAL ABSTRACTS, VOLUME 90, 1979, PAGE 295, ABSTRACT NUMBER 182705X, NUCLEIC ACIDS RES., VOLUME 6, NUMBER 2, 1979, PAGES 443-454.	1-12
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IV. CERTIFICATION		
Date of the Actual Completion of the International Search ² 24 MARCH 1983		Date of Mailing of this International Search Report ² 12 APR 1983
International Searching Authority ¹ ISA/US		Signature of Authorized Officer ²⁰ JAMES MARTINELLI